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ORIGINAL ARTICLE

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Elimination of the differential chemoresistance between the murine B-cell lymphoma LY-ar and LY-as cell lines after arsenic (As_2O_3) exposure via the overexpression of gsto1 (p28)

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Abstract *Purpose*: Arsenic, in the form of As_2O_3, has gained therapeutic importance because it has been shown to be very effective clinically in the treatment of acute promyelocytic leukemia (APL). Via numerous pathways arsenic induces cellular alterations such as induction of apoptosis, inhibition of cellular proliferation, stimulation of differentiation, and inhibition of angiogenesis. Responses vary depending on cell type, dose and the form of arsenic. GSTO1, a member of the glutathione S-transferase superfamily omega, has recently been shown to be identical to the rate-limiting enzyme, monomethyl arsenous (MMA^V) reductase which catalyzes methylarsonate (MMA^V) to methylarsenous acid (MMA^{III}) during arsenic biotransformation. In this study, we investigated whether arsenic trioxide (As_2O_3) induces apoptosis in both chemosensitive and chemoresistant cell lines that varied in their expression of p28 (gsto1), the mouse homolog of GSTO1. *Methods*: The cytotoxicity of arsenic in the gsto1- and bcl-2-expressing chemoresistant and radioresistant LY-ar mouse lymphoma cell line, was compared with that of the LY-ar's parental cell line, LY-as. LY-as cells are radiosensitive, apoptotically permissive, and do not express gsto1 or bcl-2. Cell survival, glutathione (GSH) levels, mitochondrial membrane potential, and stress-activated kinase status after arsenic treatment were examined in these cell lines. *Results*: As_2O_3 induced an equivalent dose- and time-dependent increase in apoptosis in these cell lines. Cellular survival, as measured after a 24-h exposure, was also the same in each cell line. Reduced GSH was modulated in a similar time- and dose-dependent manner. Apoptosis was preceded by loss of mitochondrial membrane potential that triggered caspase-mediated pathways associated with apoptosis. With a prolonged exposure of As_2O_3, both cell lines showed decreased activation of ERK family members, ERK1, ERK2 and ERK5. As_2O_3 enhanced the death signals in LY-ar cells through a decrease in GSH, loss of mitochondrial membrane potential, and abatement of survival signals. This effect is similar to that seen when LY-ar cells are treated with thiol-depleting agents or by the removal of methionine and cysteine (GSH precursor) from the growth medium. This response is also completely contrary to that seen for radiation, actinomycin D, VP-16 and other agents, where LY-ar cells do not succumb to apoptosis. *Conclusions*: The overexpression of gsto1 in normally chemoresistant and radioresistant LY-ar cells renders them vulnerable to the cytotoxic effects of As_2O_3, despite the 30-fold overexpression of the survival factor bcl-2. Gsto1 and its human homolog, GSTO1, may serve as a marker for arsenic sensitivity, particularly in cells that are resistant to other chemotherapeutic agents.

Keywords Apoptosis · Cell survival · Arsenic trioxide · Glutathione S-transferase omega · GSH

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Introduction

Resistance to chemotherapy is an unresolved problem in the treatment of many types of cancer. Treatment outcome varies even among the same tumor type, making it difficult to improve the management of certain cancers [1, 23]. It is known that cancer cells become resistant to chemotherapy and radiation by inhibiting apoptosis or

by upregulating survival factors, and the increased understanding of the morphological and functional characteristics of molecular signaling has led to new treatment strategies [9, 22, 25, 27]. This is certainly true for some hematological malignancies in which resistance to conventional therapies is caused by certain cells having escaped the normal developmental pathways that lead to apoptosis. For this and other reasons, a more thorough understanding of the balance between survival and apoptotic pathways and of the agents that affect these pathways is needed to develop more effective cancer treatment strategies.

Arsenical compounds (Fowler's solution) were once a mainstay of compounds used to treat a variety of diseases including chronic myelogenous leukemia. For a review of arsenicals in medicine see Waxman and Anderson [40]. However, with time arsenicals fell into disfavor because of concerns over toxicity and carcinogenicity. Still, in the 1970s Chinese physicians began to use arsenic trioxide (As_2O_3) and arsenic dioxide to treat patients with acute promyelocytic leukemia, and with great success [31, 44]. Although the precise mechanism of action of arsenic is unknown, the results of various in vitro studies have suggested that several mechanisms may contribute to its effectiveness in vivo. These mechanisms include induction of apoptosis, partial cellular differentiation, degradation of specific APL fusion transcripts, antiproliferation, generation of reactive oxygen species (ROS), and inhibition of angiogenesis [2, 4, 20, 21, 29, 38, 39]. Several studies have shown that the effectiveness of arsenic varies depending upon the valence and methylation state of arsenic and the intracellular GSH content of the cells examined [5, 7, 8, 11, 12, 28]. The requirement for GSH in the induction of apoptosis by the final metabolite of arsenic, dimethylarsonous acid (DMA), has implicated the mechanistic significance of GSH [26].

Recently, Zakharyan and Aposhian [42] have proposed that monomethylarsonate (MMA^V) reductase, which alters the methylation and valence state of arsenic, is the rate-limiting enzyme in inorganic arsenic metabolism. In many mammalian species, inorganic arsenate is first reduced to arsenite and then subsequently methylated to monomethylarsonous acid (MMA^{III}). In this pathway, MMA reductase, with an absolute requirement for GSH, catalyzes the reduction of MMA^V to MMA^{III}. MMA^{III} is further catalyzed by MMA methyltransferase to become DMA. From peptide sequence analysis, Zakharyan et al. [43] determined that MMA^V reductase is identical in sequence to the human glutathione S-transferase omega (GSTO1).

Using two mouse B-cell lymphoma cell lines, the mouse GSTO cDNA (gsto1), previously referred to as p28, had been earlier cloned via differential display [18]. And although the human cDNA had also been cloned (Genebank AG 90313), it was the work of Board et al. [3] that showed the human cDNA to be representative of GSTO1, a new class of GST. Our interest is in the cell lines from which the mouse gsto1 had been cloned. In our original studies, cells from the mouse B-cell lymphoma LY-TH were used to examine apoptosis after asparaginase exposure [33]. However, after continued culture of these LY-TH cells, they became drug- and radiation-resistant. These resistant cells were clonally isolated, as were cells from frozen cultures of LY-TH cells and the isolates were expanded and tested for radiation and drug resistance. These two B-cell lymphoma cell lines, designated LY-as and LY-ar, differ in their responses to radiation and several chemotherapeutic agents including cisplatin, Adriamycin, and etoposide (VP-16) [34, 35]. LY-ar cells are radiosensitive and chemosensitive, overexpress bcl-2 and gsto1, express NFKB constitutively as a p50 homodimer [19], have elevated levels of reduced cellular GSH [37], and do not die via an apoptotic mechanism. LY-as cells, do not express bcl-2 nor gsto1, are radiosensitive and chemosensitive, and die exclusively via apoptosis when challenged.

In previous studies, the radioresistance and chemoresistance of LY-ar cells were altered by reducing the amount of cellular thiols via short-term removal of cysteine and methionine from the culture medium [36]. This depletion of cellular thiols has been thought to be the mechanism through which LY-ar cells are made vulnerable to therapeutic agents, in spite of the overexpression of bcl-2 [24]. Given the enhanced expression of gsto1, we hypothesized that LY-ar cells, which are comparatively resistant to other chemotherapeutic agents, would be especially sensitive to arsenic even with increased levels of reduced GSH as compared to LY-as cells. Our supposition was that the presence of the relatively enhanced level of gsto1 in LY-ar cells would produce more DMA, which, in conjugation with GSH, would release more toxic metabolic byproducts, which would bring about enhanced apoptotic cell killing. To test these hypotheses, we investigated the effects of As_2O_3 in both LY-ar and LY-as cell lines by assessing apoptotic cell death and clonogenic cell survival. We also examined mitochondrial membrane potential as it is considered a major source of the putative ROS that mediates arsenic-induced apoptosis and might serve as the mechanism for arsenic sensitivity in LY-ar cells.

Materials and methods

Cell culture

The murine B cell lymphoma cell lines LY-ar and Ly-as were maintained in RPMI medium (GIBCO) supplemented with 10% fetal bovine serum (Sigma), and 2 m *M* each of glutamine, penicillin and streptomycin. Cells were maintained at 37°C in a 95% air/5% CO_2 incubator.

As_2O_3 treatment

A stock solution of As_2O_3 was made at a concentration of 10 m *M* in PBS and diluted to the appropriate con-

centration before use. In all the experiments with As_2O_3, cells were used at a concentration of 4×10^5 ml^{-1}. After different exposure times, the cells were collected by pelleting via centrifugation at 1000 *g* for 5 min. The cells were either lysed in the indicated buffer or resuspended in fresh medium for further use.

Apoptosis assay by flow cytometry

The terminal deoxynucleotidyl transferase (TdT) dUTP nick end-labeling (TUNEL) assay was used to identify DNA fragmentation (APO-DIRECT kit, Pharmingen) and was performed according to the manufacturer's instructions. Briefly, 2×10^6 cells were fixed in 1% paraformaldehyde and washed in PBS. They were then suspended in 70% ethanol and stored at −20°C until analysis. The cells were then resuspended in a staining solution containing TdT and FITC-dUTP and allowed to stain overnight at room temperature in the dark. Then the cells were rinsed and resuspended in propidium iodide/RNase A solution and analyzed by flow cytometry.

DNA fragmentation assay

Cells, 4×10^5 ml^{-1} in 20 ml, were treated with different concentrations of As_2O_3. At different times after treatment the cells were collected, and then lysed (10 m *M* Tris, 1 m*M* EDTA and 0.2% Triton X-100) while held on ice for 20 min. The fragmented DNA (supernatant) was separated from the intact chromatin (pellet) by centrifugation at 13,000 *g* for 10 min. DNA in both fractions was precipitated overnight at 4°C in 12.5% trichloroacetic acid (TCA). The precipitated DNA was resuspended in 5% TCA and hydrolysed for 20 min at 90°C. Hydrolysed DNA was allowed to react overnight with freshly prepared DPA reagent (1.5% diphenylamine, 250 m *M* sulfuric acid, and 0.01% acetaldehyde in glacial acetic acid). Absorbance at 600 nm was determined by spectrophotometry. The percent DNA fractionated was calculated from the ratio of the absorbance of the supernatant fraction to the total absorbance of both supernatant and pellet fractions.

Cell survival assay

Cell survival experiments were carried out using a modification of the protocol described by Ehmann et al. [10]. Cells (4×10^5 ml^{-1}) were treated with different concentrations of As_2O_3 for 24 h at 37°C. The cells were then resuspended in As_2O_3-free medium at a concentration of 2×10^5 ml^{-1}. Cells were returned to the incubator and allowed to grow. Regular additions of fresh medium were provided and the cultures were regularly diluted so as to keep the cell numbers and medium volumes manageable. Cell numbers in each culture were counted every other day using a particle data counter (Coulter). Cell numbers generated were derived after correcting for cell culture dilutions. The resulting set of growth curves allowed extrapolation of the exponential portion of the post-treatment growth curve back to time 0 to obtain the number of cells that had survived in comparison with the untreated cells.

GSH assay

Total cellular GSH was measured by following the method of Hissin and Hilf [13]. This method is based on the reduction of the substrate *o*-phthaldehyde (OPT), which results in a fluorescent signal. Briefly, cells were washed once with ice-cold PBS and then resuspended at 1×10^7 ml^{-1} in ice-cold lysis buffer consisting of 5% TCA, 1 m*M* EDTA and 0.1 *M* HCl (1:1:1, v/v/v). Following lysis, insoluble material was spun down at 2500 *g* for 20 min at 4°C. For the measurement of GSH, a 0.2-ml sample of the supernatant was mixed with 3.6 ml 0.1 *M* phosphate/5 m *M* EDTA buffer (pH 8.0) and 0.2 ml OPT stock (1 mg ml^{-1} ethanol) added. Fluorescence was read at 420 nm with excitation set at 350 nm. A standard curve was constructed using known quantities of GSH, which was then used to convert fluorescence readings to GSH concentrations.

Western blotting

Antibodies against mouse bcl-2, caspase-3, PARP, and Bax were purchased from BD-Pharmingen. Antibodies against *β*-actin and phospho-ERK5 were from Sigma and Calbiochem, respectively. Pan-ERK and phospho-ERK1/2 antibodies were purchased from Cell Signaling Technology. Polyclonal antibodies against gsto1 were raised as described previously [18].

At different times after As_2O_3 treatment, 20×10^6 cells were collected. The cells were washed with ice-cold PBS and then a total cell lysate was prepared by resuspending the cells in 100 µl RIPA buffer (25 m*M* Tris, pH 7.4, 150 m *M* KCl, 5 m *M* EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and a cocktail of protease and phosphatase inhibitors). The lysate was incubated on ice for 10 min and centrifuged at 16,000 *g* for 5 min at 4°C to remove the DNA and cell debris. The resulting supernatants were collected and frozen at −80°C or used immediately. Protein concentrations were quantified using the DC protein assay (Bio-Rad) following the manufacturer's protocol. Briefly, 50 µg of protein cell lysate was resolved on 10% or 12% SDS-polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore). Membranes were incubated for 1 h in a blocking solution of 5% milk in TBST buffer (10 m *M* Tris, pH 8.0, 150 m *M* NaCl, and 0.025% Tween 20). Antibodies to specific proteins were then added at a dilution of 1:1000 and incubated overnight at 4°C. After washing

with TBST, the membranes were incubated for 1 h with horseradish peroxidase-linked appropriate secondary antibodies in the blocking solution. The membranes were washed again with TBST and proteins were visualized by electrochemoluminescence (Amersham).

Mitochondrial membrane potential

JC-1 (Molecular Probes), a cationic dye, which exhibits potential-dependent dye fluorescence spectra, was used to stain mitochondria. The change in the ratio of its green to red fluorescence was determined and used to calculate the mitochondrial membrane potential. Briefly, cells were treated with different doses of As_2O_3 for 3 h. Cells were then stained with medium containing JC-1 (10 μg ml^{-1}) at 37°C for 20 min. Following incubation, cells were washed with PBS, resuspended in PBS and analyzed by flow cytometry for red and green fluorescence.

ICP-MS and IC-coupled ICP-MS analysis of arsenic species

Quantification of different species of arsenic and inorganic arsenic was done following the protocol described by Kala et al. [17]. Briefly, cells were treated with different concentrations of arsenic for 1 h, harvested, and washed with ice-cold PBS. Cells were sonicated in 0.25 *M* ice-cold perchloroacetic acid (PCA) and centrifuged at 16,000 *g* for 10 min. The arsenic content in the supernatant was quantified using a standard calibration curve of inorganic arsenic (SPEX, Metchen, N.J.) with terbium as an internal standard. Total arsenic content in samples was expressed as nanograms per milligram protein. A portion of the samples was used for arsenic speciation using IC/ICP-MS (HP4500). Arsenic species (DMA^V, As^{III}, MMA^V and As^V) were separated on a Dionex anion exchange column (AS7, Dionex, Houston, Tx.), with a gradient containing 30 m *M* ammonium acetate (pH 9.0), 30 m*M* ammonium phosphate (pH 4.5), 200 m *M* ammonium hydroxide, and water. The flow rate of the column was maintained at 1.5 ml min^{-1}, and the arsenic content in the effluent was continuously monitored by ICP-MS. Standard chromatograms were generated using commercially available DMA^V, As^{III} (sodium arsenite), MMA^V and As^V (sodium arsenate) (Sigma) to identify and quantify the arsenic species.

Results

The promising use of arsenic as a novel anticancer drug for malignant lymphoid cells and solid tumors is based partly on its ability to induce apoptosis. To test whether arsenic is capable of overcoming the impervious nature of LY-ar cells, particularly in light of the overexpression of gsto1, the induction of apoptosis was examined using two methodologies to measure fragmented DNA. Arsenic induced apoptosis in both LY-ar and in LY-as cells. As shown in the Fig. 1, flow cytometric analysis revealed that upon treatment with As_2O_3, LY-ar cells underwent apoptosis in a time- and dose-dependent manner to the same extent as LY-as cells. This is unlike with other agents such as cisplatin, Adriamycin, etoposide, or γ-irradiation (see Fig. 2 for the apoptotic and survival response to these compounds) where there is a distinctly different apoptotic response that is manifested at the cellular survival level [36].

To confirm that arsenic induces cell death through an apoptotic mechanism, DNA fragmentation, the hallmark of apoptosis especially for LY-as cells, was measured in both cell lines. Figure 3 shows time-dependent and dose-dependent increases in DNA fragmentation after As_2O_3 treatment in both LY-ar and in LY-as cells. It should be noted that the calculated percent-fragmented values are lower than the percent apoptosis values obtained by flow cytometry, which can be explained by the limitations of the DNA fragmentation technique used in this study.

The level of gsto1 protein at different time points after a treatment with arsenic at 2 μ*M* was checked to determine if As_2O_3 exposure caused any change in the expression of gsto1. As shown in Fig. 4, As_2O_3 did not alter the level of gsto1 in either cell line as compared to their respective controls; that is, there was neither upregulation of gsto1 in LY-as cells, nor degradation of gsto1 in LY-ar cells.

If gsto1 is responsible for enhanced apoptotic cell death in LY-ar cells through the production of DMA, one would expect an enhanced conversion in LY-ar cells as compared to LY-as. Table 1 shows the uptake of total arsenic, the production of DMA for both cell lines for either two or three concentrations of As_2O_3 exposure, and the percent of arsenic converted to DMA. LY-ar cells converted, on average, twice as much arsenic to DMA as LY-as cells over the 1-h exposure time. That LY-as cells convert arsenic to DMA would seem paradoxical since LY-as cells do not express gsto1 protein to any great degree, as measured by Western blot. However, all LY-as cell cultures have contaminants of LY-ar cells, there is a spontaneous conversion to the resistant LY-ar phenotype with continued cell culture. This may be at least partially responsible for the DMA seen in LY-as cells.

The apoptotic phenotype is induced by the activation of constitutively expressed proteins which include a family of cysteine proteases. To characterize the molecular pathways involved in arsenic-induced apoptosis, we performed Western analysis of different apoptotic proteins. As shown in Fig. 4, As_2O_3 treatment activated caspase 3 and PARP cleavage in both LY-ar and LY-as cells. Although the appearance of the 17 kDa form of active caspase 3 could be seen as early as 3 h after arsenic treatment in LY-ar cells and 6 h in LY-as cells, we did not see any change in the level of procaspase 3 in

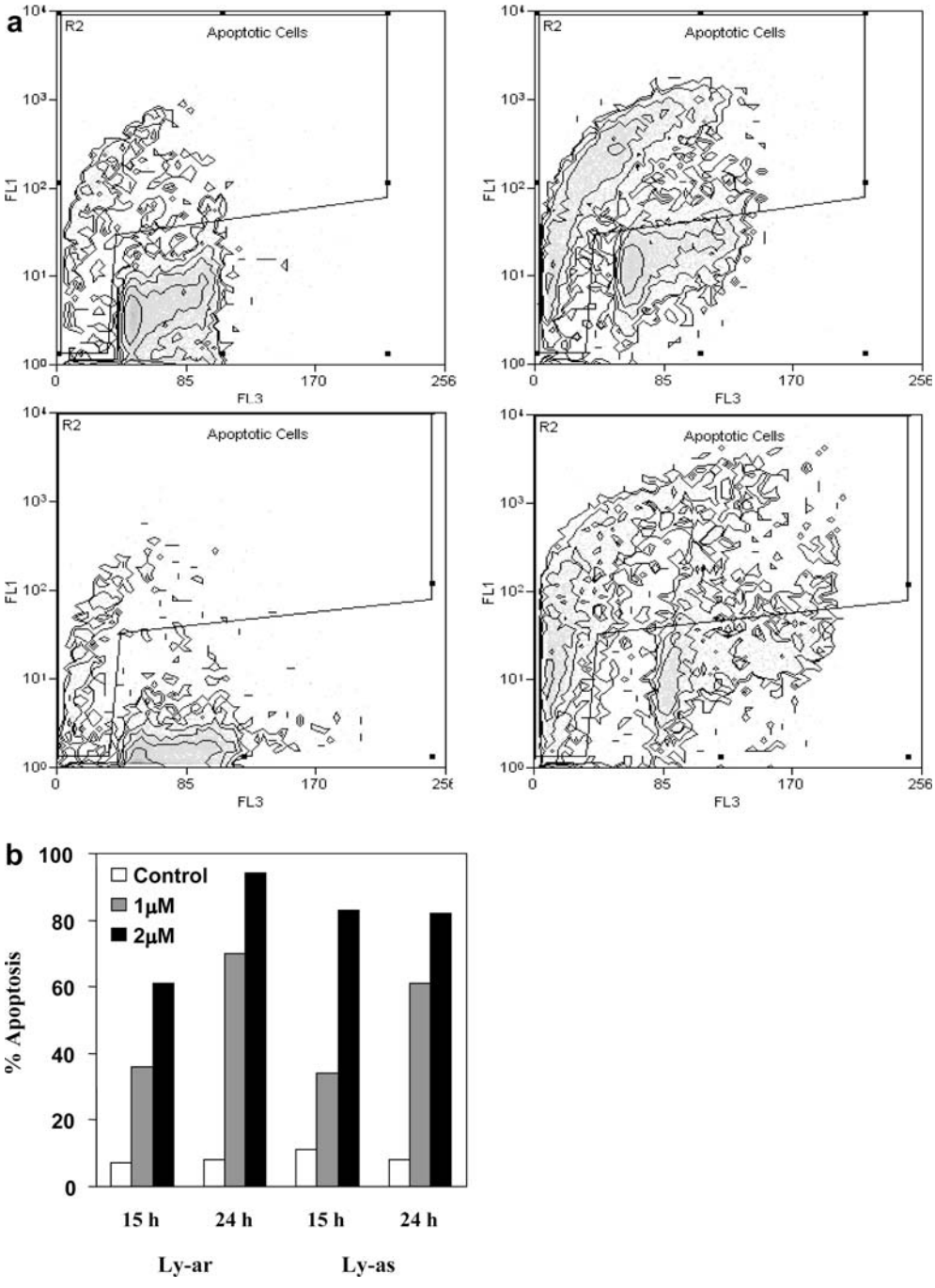
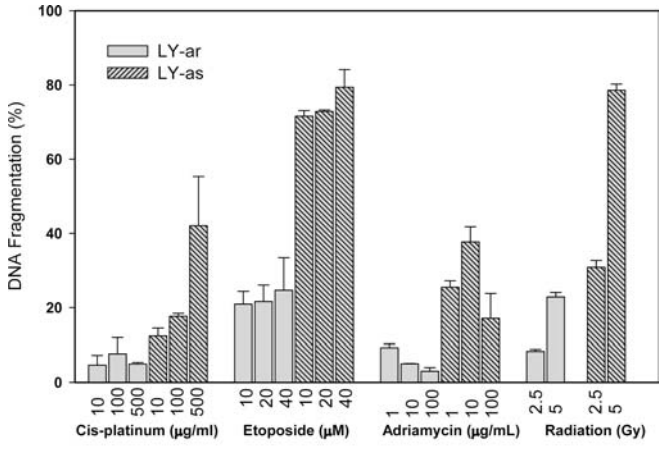


Fig. 1 **a** Effect of As_2O_3 on the induction of apoptosis in LY-as and LY-ar cells. Apoptosis was measured by flow cytometry after treating the cells with 1 μ*M* As_2O_3 for 24 h. *FL3* is linear integral red fluorescence (PI), *FL1* is integral log of green fluorescence (FITC). Signals were pregated to exclude clumps and doublets. Each panel represents percent apoptosis: *upper left* LY-as control (8%), *upper right* LY-as treated with As_2O_3 (61%), *bottom left* LY-ar control (8%), *bottom right* LY-ar treated with As_2O_3 (70%). **b** As_2O_3-induced apoptosis as measured by flow cytometry. Ly-ar and LY-as cells were treated with 1 μ*M* and 2 μ*M* As_2O_3 for the indicated times. Following treatment cells were fixed in 1% paraformaldehyde and stained as described for flow cytometric analysis

either cell line (data not shown). The downstream effector molecule PARP was cleaved into an 89 kDa fragment, which increased as full-length PARP decreased with time, both in LY-ar and LY-as cells. The levels of both forms of PARP were reduced significantly by 24 h in both cell lines. Although there was no significant change in the levels of bcl-2 in LY-ar cells, the appearance of a cleaved product of about 19 kDa was observed upon As_2O_3 treatment suggesting the active involvement of proteases to effect As_2O_3 toxicity. Interestingly, there was no change in the level of Bax, another key component for stress-induced apoptosis, in either cell line. Bax upregulation is normally seen in LY-as cells upon irradiation or exposure to other chemotherapeutic agents [26].

In spite of the overexpression of bcl-2 in LY-ar cells and its normal inhibition of apoptosis, treatment with As_2O_3 for 24 h decreased survival of LY-ar cells to the levels seen for LY-as cells (Fig. 5a). As shown in Fig. 5b, relative survival was decreased significantly with As_2O_3 exposure suggesting that the enhancement of apoptosis is reflected in cellular survival for LY-ar cells.

Cellular GSH content has been implicated in many cellular responses to arsenic [8, 38]. In order to deter-



Drug Treatment	IC_{50}		IC_{10}	
	LY-ar	LY-as	LY-ar	LY-as
Cis-platinum (μg/ml)	1.8	1.0	5.22	3.15
Etoposide (μM)	0.22	0.13	0.93	0.60
Adriamycin (μg/ml)	0.033	0.014	0.088	0.048
Arsenic Trioxide (μM)	0.625	0.65	0.10	0.08
	LD_{50}		LD_{10}	
Radiation (Gy)	.066	.036	2.15	1.2

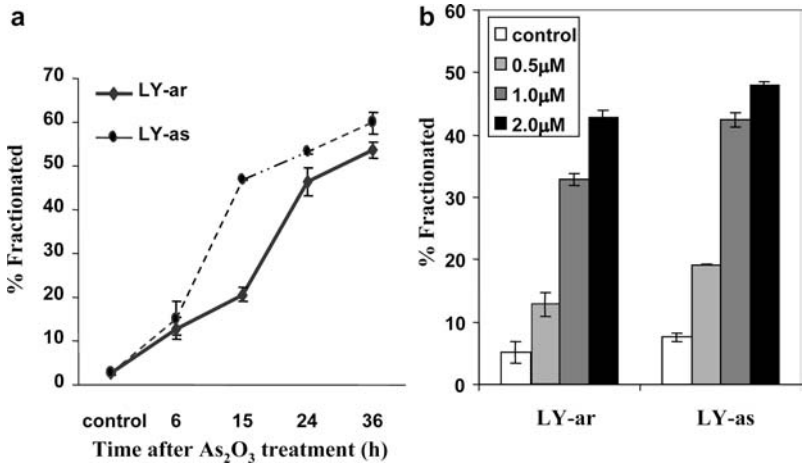
Fig. 2 Apoptosis induced in LY-ar versus LY-as after exposure to several toxic agents. Apoptosis was measured as percent DNA fragmentation of the labeled DNA. Drug exposures were for 1 h and apoptosis was measured 3 h later. Apoptosis after radiation was measured 4 h after exposure (^{137}Cs, 4 Gy min^{-1}). The underlying table shows IC_{50} and IC_{10} values for cell survival after drug exposure, and LD_{50} and LD_{10} values for radiation exposure. IC_{50} and IC_{10} values for cisplatin, etoposide and Adriamycin were derived from Story and Meyn [36]. Arsenic values were derived from Fig. 5

Fig. 3 **a** Time-dependent effect of As_2O_3 on DNA fragmentation in LY-as and LY-ar cells. Cells were treated with 2 μ*M* As_2O_3 and harvested at the indicated time points and processed as described in Materials and methods. Percent-fractionated is the direct measure of DNA fragmentation induced by As_2O_3. *Error bars* denote SE from three different experimental samples. **b** DNA fragmentation in LY-as and LY-ar cells as a function of As_2O_3 concentration. Cells were treated with the indicated concentrations of As_2O_3 for 24 h and processed as described in Materials and methods. *Error bars* denote SE from three different experimental samples

mine whether the differential levels of GSH in LY-ar and LY-as cells are modulated by arsenic, we measured the intracellular content of GSH at 6 h and 15 h after treatment with increasing doses of As_2O_3. As shown in Fig. 6, with As_2O_3 at 1 μ*M* there was a moderate increase in cellular GSH content for up to 15 h in both cell lines. Although 6 h (Fig. 6a) treatments with As_2O_3 at 2 μ*M* and 5 μ*M* did not significantly change the levels of GSH in LY-ar cells, and a 15-h (Fig. 6b) treatment at 5 μ*M* diminished the level of GSH to less than 25% of the control value in LY-ar cells and completely depleted GSH in LY-as cells.

Since it is known that any imbalance in redox potential can modulate GSH [15], we tried to determine whether arsenic mediates this imbalance by generating ROS through altered mitochondrial membrane potentials. We used a flow cytometric measurement of JC-1, whose fluorescence is dependent upon the membrane potential of mitochondria. Figure 7 shows a dose-dependent increase in percent depolarization of mitochondria in both cell lines 3 h after As_2O_3 treatment. Although LY-ar cells showed lower percent depolarization values than LY-as cells, depolarization increased with increasing dose. This lower extent of depolarization in LY-ar cells likely reflects the data of Fig. 6 where at early times GSH levels had not yet fallen.

We further investigated the effect of arsenic-induced ROS on the activation of stress-related kinases, in particular the ERK family members, by performing Western analysis with phospho-specific antibodies. Figure 8 reveals that As_2O_3 inhibited the activation of ERK5 as early as 3 h and total inhibition could be seen by 15 h for both cell lines. Although As_2O_3 exposure did not change the activation of ERK1 in either cell line, by 3 h ERK2 activation was enhanced in both cell lines and it remained elevated for at least 15 h after treatment. However, by 24 h exposure ERK2 activation was found to be at or near control levels in LY-ar. LY-as data were not available for this time after As_2O_3 treatment because most of the cells were rendered to apoptotic bodies making it difficult to obtain enough protein for analysis.



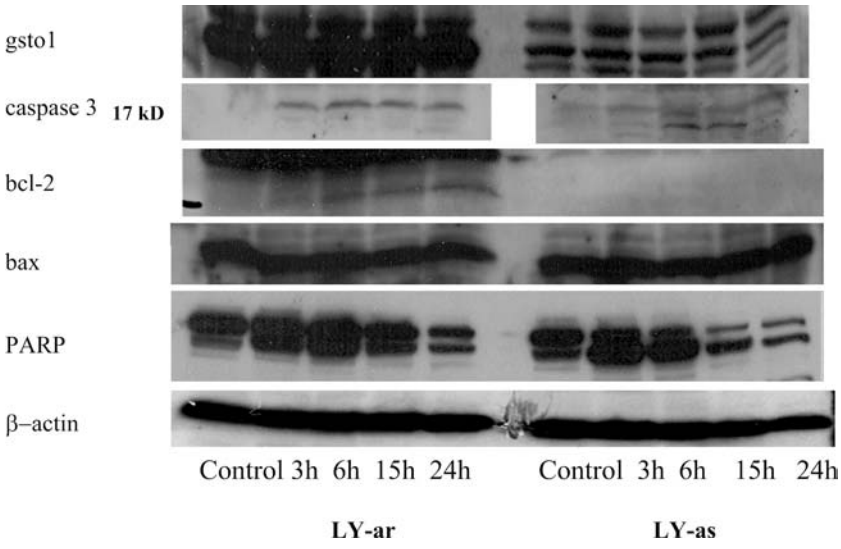


Fig. 4 Effect of As_2O_3 on the levels of proteins involved in apoptotic process and on gsto1. Ly-ar and LY-as cells were treated with 2 μ*M* As_2O_3 and at the indicated time points cells were collected, washed with PBS and total cell lysates were prepared. The protein, 50 μg from each sample, was resolved on 12% SDS-PAGE for analysis by Western blotting. *β*-Actin was used as a loading control. Results are representative of three separate experiments

Discussion

Chemoresistance and radioresistance are thought to be two of the major causes of treatment failure and death among patients with cancer. Knowledge gained about drug resistance mechanisms can be applied in the clinic to stratify patients, but it is important to ascertain the relevance of in vitro mechanisms of resistance to clinical practice. Apoptosis is one such mechanism and it is dependent on the intracellular balance between proapoptotic and antiapoptotic signals. For many cancer cells, the response to a particular chemotherapy agent depends on the extent of the perturbation of this balance.

In this study, we showed that a murine B-cell lymphoma cell line, LY-ar, which is comparatively resistant to apoptosis induced by radiation and nearly all other chemotherapeutic agents tested, undergoes massive cell death via apoptosis in response to As_2O_3 treatment. The mechanisms of arsenic-induced apoptosis in LY-ar cells were similar to those in its parental cell line LY-as, which is non-bcl-2-expressing, radiosensitive and chemosensitive, and apoptotically permissive. The relative survival of these cell lines is reflected in the extent to which apoptosis occurred. Downregulation of bcl-2 has been implicated in arsenic-induced apoptosis in many cell lines [4] and it is interesting that overexpression of bcl-2 failed to protect the LY-ar cells against arsenic-induced cell death. Indeed, cleavage of bcl-2 was seen in As_2O_3-treated LY-ar cells. Although the level of another key component of apoptosis, Bax, was not altered in these cell lines, it may be participating in arsenic-induced apoptosis by oligomerization and translocation. PARP cleavage clearly correlated with the time-dependent increase in arsenic-induced apoptosis in both cell lines as did the activation of caspase 3.

The levels of gsto1 (MMA^V reductase) were not altered by arsenic treatment in either cell line. Interestingly, we have yet to see any upregulation of gsto1 in LY-as in response to As_2O_3 or any other agent, and LY-ar cells have the highest basal level of gsto1 we have seen in any cell line. In that case gsto1 may not be subject to upregulation. Still, we have seen upregulation of gsto1 in response to radiation (manuscript in preparation) in other cell lines.

One might expect the GST, thiol transferase, or ascorbate reductase activities of gsto1 to be involved in counteracting the effects of arsenic. GSH has been shown to play a major role in biotransformation, cytotoxicity, and the excretion of arsenic [8, 16, 26]. GSH is a

Table 1 Comparison of arsenic conversion to DMA in LY-ar and LY-as cells. LY-ar cells were exposed to 5, 10, and 20 μ*M* As_2O_3, while LY-as cells were exposed to 10 and 20 μ*M* As_2O_3 for 1 h. Correcting for the initial concentration of As_2O_3 used shows that the percentage of DMA produced in LY-ar cells was twofold higher

	As treatment (μ*M*)	Total As intake (ng ml^{-1})	DMA (ng ml^{-1})	Total As (ng mg^{-1} protein)	DMA (ng mg^{-1} protein)	Percent As converted to DMA	Average percent conversion ± SEM
LY-ar cells	5	107	63.16	10.99	6.48	59.03	50.73 ± 7.02
	10	229	84.21	23.23	8.54	36.77	
	20	280	157.89	23.73	13.38	56.39	
LY-as cells	10	162	44.74	19.95	5.51	27.62	26.44 ± 1.18
	20	250	63.16	32.98	8.33	25.26	

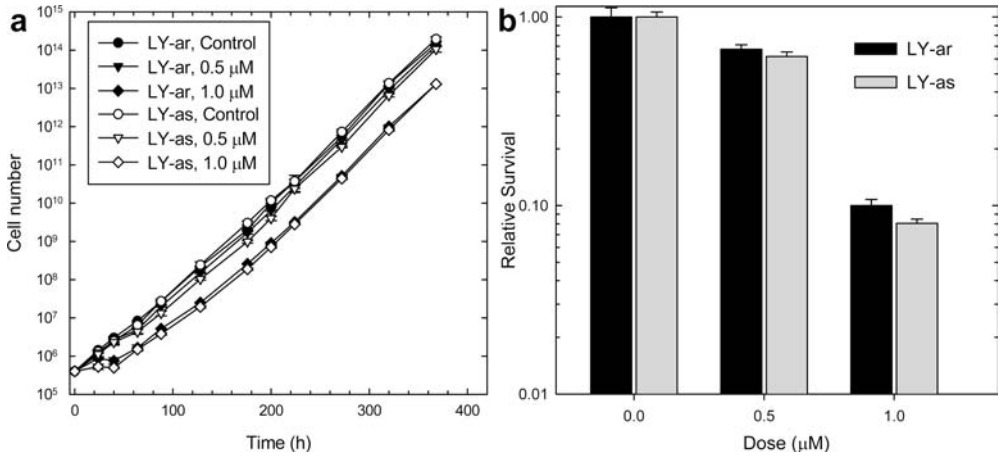


Fig. 5 Survival of LY-as and LY-ar cells after As_2O_3 treatment. **a** Effect of As_2O_3 on cell division in LY-ar and LY-as cells. Cells were treated with 0.5 μ*M* and 1 μ*M* doses of As_2O_3 for a period of 24 h. Following treatment cell counts were obtained. Collected cells were maintained in As_2O_3-free medium. Cell counts obtained by Coulter counter are plotted as a function of time. Each cell count is the mean of duplicate samples. **b** Relative survival. Relative survival was calculated based upon the back-extrapolation of the linear portions of the growth curves of **a**

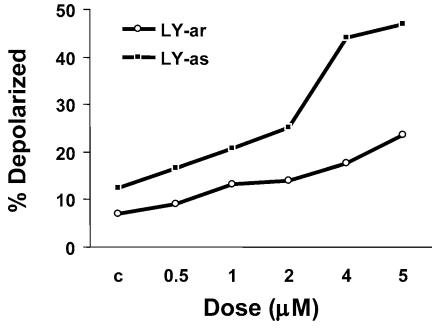


Fig. 7 Effect of As_2O_3 on mitochondrial membrane potential in LY-ar and LY-as cells. Mitochondrial membrane potential was determined by flow cytometry after incubation with the indicated doses of As_2O_3 for a period of 3 h. After incubation, cells were stained with JC-1 and the florescence change was analyzed by flow cytometry. The results presented are the mean values of two experimental samples

ubiquitously expressed thiol that squelches free radicals, detoxifies electrophilic compounds through GST-mediated reactions, and helps to maintain a normal redox state. A relationship between the levels of GSH and arsenic sensitivity has been implicated in many cell types [38, 41]. In this study, the observed effect of lower doses of arsenic on the levels of GSH in both LY-ar and LY-as cell lines illustrates that the initial increase in GSH is likely to exert an antioxidant effect that is eventually diminished by prolonged exposure to arsenic. With higher doses of arsenic a rapid decline in the level of GSH was seen and the depletion of GSH is likely required for the full effect of As_2O_3-induced apoptosis and subsequent cell death. In several studies with LY-ar cells, we have overcome the impervious nature of these cells by depleting thiols [24, 36]. However, in this study arsenic exerted its effect without any need for a thiol-depleting agent.

Modulation of GSH levels is expected to result in morphological and functional changes in mitochondria. The effect of a 3-h As_2O_3 exposure on the depolarization of mitochondrial membrane potential illustrates that arsenic can directly impair the mitochondria. The lower percent depolarization values for all the doses in LY-ar cells as compared to LY-as cells can be explained by their differential constitutive levels of GSH, particularly at the early time examined, and may have been reflective of GSH levels shown in Fig. 6. The impairment of mitochondria may further be potentiated by the arsenic-

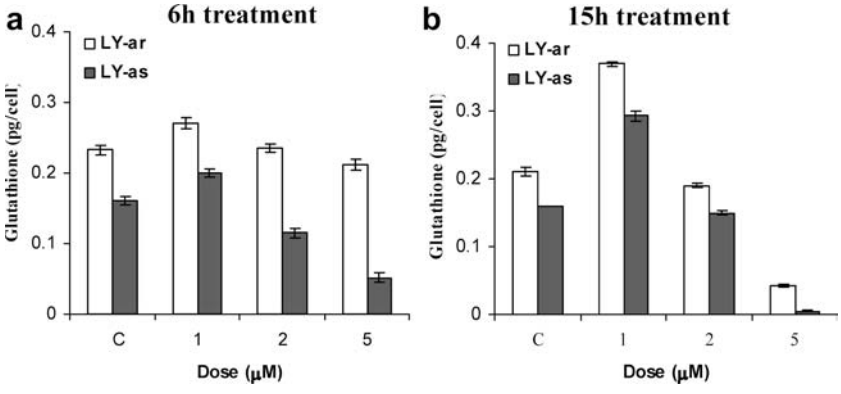


Fig. 6 Effect of 6 h (**a**) and 15 h (**b**) treatment with As_2O_3 on the levels of total cellular GSH in LY-as and LY-ar cells. Cells were treated with the indicated dose of As_2O_3 and the levels of total cellular GSH were measured. The results presented are the means ± SE of three different experimental samples

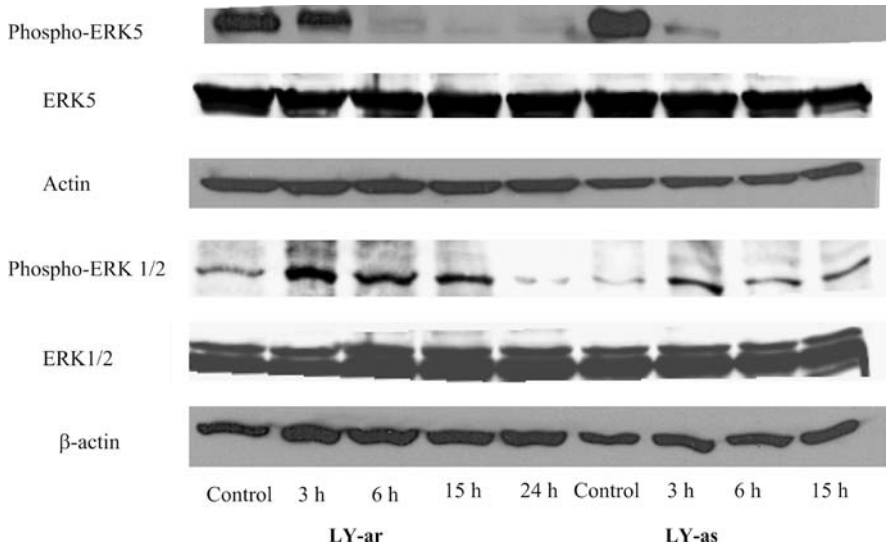


Fig. 8 Effects of treatment of LY-ar and LY-as cells with As_2O_3 on the levels of ER kinases and their activated (phosphorylated) forms. Cells were treated with 2 μ*M* As_2O_3 for different time periods. 75 μg (for the phosphorylated form) or 50 μg (for the native form) of protein from the total cell lysate, prepared as described in Materials and methods, was resolved on 10% SDS-PAGE and subjected to Western analysis using the indicated antibodies

induced depletion of GSH levels. Downstream effects caused by disruption of the mitochondrial membrane can be seen in the activation of cysteine proteases associated with the apoptotic pathway. All these findings together suggest that arsenic is able to overcome the resistance of LY-ar cells to apoptosis by modulating intracellular GSH and disrupting mitochondrial membrane potential. The findings of a more recent study by Hu et al. [14] in MOLT-4 and its daunorubicin-resistant cell line are similar.

Because several studies have shown a strong association between arsenic and skin/urinary bladder cancer [6, 30, 32], we further studied the effect of arsenic in cell survival pathways, especially those involving the stress-activated kinases. In both LY-ar and LY-as cells treated with As_2O_3, we observed the appearance of phosphorylated ERK2, which is considered a survival signal. However, with prolonged arsenic treatment (24 h), the level of phosphorylated ERK2 returned to control level in LY-ar cells. On the other hand, the decline in phosphorylated ERK5 was far more rapid, being eliminated in both cell lines within 6 h. With As_2O_3 exposure not only are death signals in LY-ar cells enhanced, but survival signals are also reduced to insure the effect of the apoptotic cascade.

The role played by gsto1 in the arsenic-induced apoptosis of LY-ar cells is not completely established. However, from this study it is evident that the LY-ar cell line, which is resistant to radiation and many chemotherapeutic drugs, underwent apoptosis and survival inhibition with arsenic treatment to approximately the same extent as the parental LY-as cell line. The differential in apoptosis seen is modest when other agents, as depicted in Fig. 2, are examined, and one could argue that the extent of apoptosis in LY-ar cells is trailing that of LY-as cells because of the increased basal level of GSH in LY-ar cells. As would be expected, when cell survival was examined, there was no real difference in survival outcome. Interestingly, with As_2O_3 or with other drugs where thiols were depleted in order to effect sensitization of LY-ar cells to radiation or chemotherapeutic agent, the sensitivity of LY-ar cells has never been made greater than that of LY-as cells [36]. In order to make a direct link between gsto1 and the As_2O_3 response of LY-ar cells, derivatives of LY-ar and LY-as cell lines or other cellular models where gsto1 can be modulated need to be created. Successful transfection or infection of LY-ar cells has yet to be accomplished. This work is ongoing, as is the examination of clinical leukemia and lymphoma samples for the incidence of overexpression of GSTO1 in human cancers, where the expression of GSTO1 may serve as a marker for arsenic sensitivity and perhaps serve as a tool for treatment strategy decisions. One could envision the addition of As_2O_3 to clinical regimens in cases where GSTO has been shown to be overexpressed in tumor cells and where tumor cells are refractory to standard drug therapy regimens.

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